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Oxidative DNA Damage Is Reduced Following a Novel 3-Month Supplementation Intervention in Hemodialysis Patients

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Abstract

Chronic renal failure patients receiving haemodialysis (HD) exhibit a high incidence of cardiovascular disease and cancers. This is reported to be attributed to elevated levels of genetic damage coupled with lower levels of antioxidants, both endogenous and exogenous, and thus higher levels of oxidative stress. In the UK, HD patients are not prescribed supplementation, unlike other countries. This study is a blinded randomised intervention where 38 HD patients were assigned either placebo or novel supplement for 3 months. The modified comet assay was used to measure levels of DNA damage. The % of tail DNA damage was used to measure basal genetic damage; oxidative-specific DNA damage was measured with the addition of the enzymes Endo III and FPG. The HD patients receiving treatment had significantly reduced levels of all types of DNA damage compared to the placebo at 3 months. We observed a positive correlation between the duration on dialysis (months) and levels of Endo III -specific damage ($p=0.041$). Finally, in the HD placebo group, DNA damage levels were significantly increased from baseline at 3 months. This supplement, which is not available in the UK, may offer a treatment to reduce DNA damage, thereby helping to reduce the impact of HD on genomic damage and thus, cancers and CVD. As such, it warrants further investigation.

Keywords: DNA; Chronic renal failure (CRF); Reactive nitrogen species (RNS)

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Introduction

Chronic renal failure (CRF) has been described by [1] as a multifactorial immuno-inflammatory syndrome; this condition worsens as patient's progress to maintenance dialysis therapy one treatment for CRF is haemodialysis (HD), however, the interaction between the HD patient's blood and the semipermeable membranes contained in the haemodialysis apparatus increases levels of oxidative stress in these patients [2]. Indeed, during this interaction, circulating neutrophils are responsible for generating reactive oxygen species (ROS), such as superoxide, which further enhances levels of oxidative stress [3]. In addition to the damage from the dialysis membrane, HD patients recruit inflammatory cells, including neutrophils and macrophages, to the damaged parts of the kidney. Their oxidant-generating enzymes, such as nitric oxide and NADPH oxidase, produce high concentrations of ROS-and reactive nitrogen species (RNS) [4.] Excessive, long-lasting levels of ROS leads to metabolic dysregulation and /or the oxidation of end products including proteins, lipids, and DNA and/or oxidative damage in cells, tissues or organs [5,6] The resulting proliferation of

oxidative stress leads to an increase in DNA damage in patients undergoing HD [7] We have also reported that HD patients have increased levels of oxidative DNA damage compared to healthy individuals [8]

Several studies have reported an increased risk of developing cancer in patients with CKD than in the general population [9,10,11] and in a retrospective cohort study, by Lee et al, [12], who reported an increased risk of hepatocellular, kidney, bladder, extra kidney/bladder urinary tract, and thyroid cancers in dialysis patients. In addition, Schupp *et al.*, [13] suggest that end-stage renal disease (ESRD) patients have an increased risk of developing cancer as a result of a prolonged uremic state, chronic infection, lowered immune system, nutritional insufficiencies and altered DNA repair.

HD patients are required to follow a restricted diet, which may lead to malnutrition in between 40-50% of these patients [14]. An impaired antioxidant system has been reported in HD patients with particular focus on serum selenium, vitamins C and E levels and activities of glutathione peroxide, catalase and superoxide dismutase, all have been reported to be lower than control participants [15-18]. These factors, acting alone or in

combination, further compromise these patients and may lead to the elevated levels of ROS and increased level of cancers in this patient group.

The European Standards Committee on Oxidative Damage (19) recommended that the comet assay was the most accurate method for measuring DNA damage within eukaryotic cells. This is a sensitive, single gel electrophoresis technique for detecting DNA strand breaks at the level of individual cells. Furthermore, the comet assay has various modifications to detect specific base alterations [20]. One such modified assay uses the bacterial enzymes to identify oxidative specific damage; endonuclease III (Endo III) and Formamidopyrimidine DNA Glycosylase (FPG) which identify pyrimidine- pyrimidine breaks and purine-purine breaks respectively [21]. This therefore enables the comparison of oxidative specific damage at baseline and post study.

Given the impaired antioxidant status in HD patients outlined above, supplementation with antioxidants has been suggested as a treatment to reduce cardiovascular mortality and morbidity in CKD patients however, in a Cochrane Review [22] the authors reported no clear overall effect on cardiovascular mortality however, supplementation in pre-dialysis CKD patients may prevent progression to ESKD.

Supplementation with Vitamin E has been reported by several authors [23-26] to be beneficial and they report a reduction in PUFA peroxidation. In addition, clinical trial results have also shown positive results with Vitamin E supplementation in HD patients [27].

Supplementation with Vitamin C, Vitamin E and catalase have been reported to show small reductions or no effect on oxygen radical generated DNA Damage using the comet assay [28]. Given these data we conclude that the benefits of using a single antioxidant supplementation regime in HD patients, still remain controversial. This may be due the abnormalities do not seem to respond readily to single intervention strategies such as folic acid, Vitamin C, or antioxidant treatment alone [29-31].

Therefore, we have designed a micronutrient supplement of physiological doses of trace elements and antioxidant vitamins to investigate whether replenishment of these could counteract the actions of ROS and therefore decrease the levels of oxidative DNA damage in HD patients.

Materials and Methods

Participants

Thirty-eight individuals undergoing HD at the Western Health and Social Care Trust (WHSC) were recruited to the study following informed consent. Ethical approval was obtained from the Office of Research Ethics Committees, Northern Ireland (ORECNI) and Research Governance approval from WHSC. All procedures followed were in accordance with the ethical standards of the committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Exclusion criteria included individuals who were smokers, had known alcohol abuse in the preceding 12

months, severe liver disease, severe uncontrolled cardiac or respiratory disorder, pre-existing malignancy, pregnancy or breast feeding and already recruited to other clinical trials. Any volunteers who had taken vitamin supplements prior to baseline, had a four week wash out period before the study started. Once participants had provided informed consent they were enrolled onto the study. Each participant was given a unique study identifier on enrolment, all data was treated in a confidential manner, with hard copies stored in a locked filing cabinet at Ulster University and electronic data protected by a unique password.

Routine Blood Sampling

As part of the routine clinical management of HD, all patients provide monthly blood samples; while the routine sample was being collected an additional 8ml blood, was collected into lithium-heparin coated vacuttes for analysis of DNA damage.

Comet Assay

The protocol used was first described by Singh *et al.* [32]. and included the modifications described by Collins *et al.* [33]. In summary, approximately 100,000 cells were mixed well with 75µl of 0.5% low melting point agarose. This mixture was then pipetted onto the slides pre-prepared with normal melting point agarose gels. For each blood sample, a total of four gels were prepared. These slides were left to solidify in a fridge (4°C) for at least 15 minutes. Fresh lysing solution was prepared (using 1% Triton-X, 10mM Tris, 10mM Na₂-EDTA and 2.5M NaCl). The slides were left in lysis solution overnight, in a container which blocks out light. Slides were then washed three times in calcium and magnesium free PBS, for five minutes each time. After this, the gels were treated with 20µl of either FPG, Endo III Buffer, or Endo III enzyme. One slide was left untreated, to be used as a blank. These were left for 45minutes at 4°C before being horizontally placed in an electrophoresis chamber, and submerged in freshly prepared electrophoresis buffer (1mM EDTA & 300mM NaOH at pH 13). Slides were left in this buffer for 20 minutes to allow for unwinding of the DNA with exposure of alkali-labile sites. Electrophoresis was then carried out at 25V and 300mA for 20 minutes. After this, the slides were removed from the electrophoresis chamber and washed in neutralising solution (0.4M Tris, pH 7.5) three times for five minutes each. Each gel was then stained with 20µl of ethidium bromide (20µl/ml) and coverslips placed on the slides before analysis under the fluorescence microscope.

Throughout this study, % DNA in the comet tail was used as the parameter to determine levels of DNA damage, i.e. the % of total nuclear DNA that migrated to the tail. Fifty comets per gel were analysed, and the mean percentage of DNA in the tail was used to indicate the frequency of DNA breaks. The % tail DNA in untreated cells (buffer only) can be subtracted from the % DNA in the tails of cells with enzyme (FPG/ENDO III enzyme) to give the net amount of damage represented by each of these enzymes. This has been reported as the net oxidative-specific DNA damage.

Image Analysis of Cells

Within one hour of staining with ethidium bromide, the cells were observed using a Nikon Optiphot compound microscope fitted with a Nikon Fluor objective of 0.85 of numerical aperture and an epifluorescence mercury lamp. The Komet 5.5 Image Analysis System was used, and the cells were viewed at a magnification of x 40. Fifty randomly chosen cells were analysed per slide, and results were given as % tail DNA for the levels of DNA damage.

Intervention

This was a double blinded study where participants were randomised, on the basis of their baseline homocysteine (tHcy) level, to receive either micronutrient supplement or placebo for 3 months. Supplements were prepared by the Pharmacist in WHSCT and administered weekly to the participants. And used boxes collected, records were kept of un-used pills and participants were only included in the analysis if they had taken 90% of the supplement. The micronutrient supplement contained: Folic acid, 800µg; Vitamin B6, (10mg); Vitamin B12, (12µg); Thiamin, (B1) 1mg; Riboflavin (B2), 1.6mg; Pantathenic acid (B5), 1mg; Vitamin C, 60mg; Vitamin E ,10mg; Vitamin K, 65µg; Zinc, 15mg; Copper,1.5mg; and Selenium, 75µg (Biosynergy, London, UK). Placebo contained:

Statistical Analysis

This was carried out using version 26.0 of the Statistical Packages for Social Sciences (SPSS) and Microsoft Excel. Data are expressed as mean ± standard error. A paired Student t-test was employed to compare differences between treatment vs treatment and placebo vs placed at baseline and post treatment; while and unpaired t-test was used to compare placebo and treatment post treatment. Pearson’s product-moment coefficient was used to assess any correlation between levels of DNA damage post treatment and age, gender, BMI, duration of dialysis and diabetes. A P-value <0.05 was set to be statistically significant.

Results

Baseline characteristics of all participants in the two experimental groups are presented in Table1. No significant differences were observed at baseline between placebo and treatment groups. Thirty-eight haemodialysis patients enrolled but only 30 completed the study. Reasons for withdrawal included: enrolment on another study, receiving a transplant and several participants passed away during the study.

Table 1: Baseline Participants Characteristics.

Characteristic	Placebo group (n=19)	Treatment group (n=18)
Age (Years)	62.85 ± 10.95	64.89 ± 8.29
Male/Female (n/n)	7-Dec	8-Oct
Diabetes (n (%))	6 (31.6%)	7 (38.9%)
Dialysis Duration (months)	27.00 ± 17.75	27.33 ± 38.09
BMI (kg/m ²)	27.08 ± 6.43	26.29 ± 4.80

BMI: Body Mass Index; Values are presented as mean ± Standard deviation.

The comet assay was used to measure general DNA damage i.e., Alkaline damage no enzymes and levels of oxidative DNA damage in placebo and treatment groups at baseline and post-intervention. FPG and Endo III net specific oxidative DNA damage, was calculated by subtracting the buffer only DNA damage levels from the FPG and Endo III damage levels.

We observed a significant reduction in DNA damage (all types) in the treatment group post intervention, compared to baseline (Figure 1). While in the placebo group DNA damage was significant higher at post intervention (**p>0.00; *p>0.05) compared to baseline in all DNA damage types (Table 2).

Net Oxidative specific DNA damage, FPG and Endo III was significantly reduced in the treatment group, (* p>0.05; **p>0.001, respectively) post intervention compared to baseline (Figure 1), while in the placebo group FPG was significantly higher (p>0.05) post intervention and Endo III was lower but did not reach significance.

In addition, we observed a positive correlation between the duration on dialysis (months) and levels of Endo III specific damage (p=0.041).

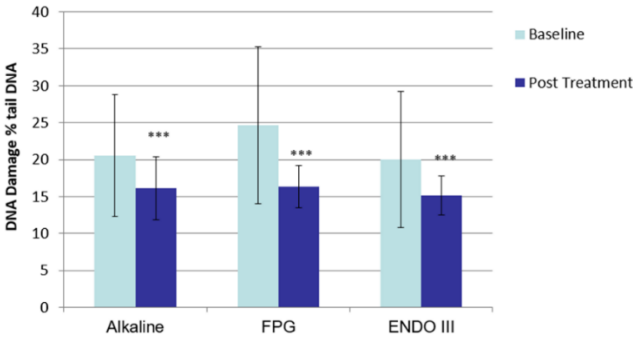


Figure 1: DNA Damage in Treatment Group Pre- and Post-Intervention. Values are mean ± SD ***p>0.001

Table 2: DNA Damage in Placebo Group Post-Intervention.

% Tail DNA	Alkaline	FPG	ENDO III
Baseline	22.22 ± 8.96	23.24 ± 9.02	22.37 ± 10.28
3 Months	37.27* ± 8.11	30.44** ± 9.77	35.26*** ± 9.32

Values are mean ±SD ***p>0.001; **p>0.01 * p>0.05.

Table 3: Net Oxidative specific DNA damage in Treatment and Placebo Groups.

DNA Specific Damage (%Tail DNA)		FPG	ENDO III
Placebo	Baseline	5.92 ± 5.46	5.87± 5.68
	3 Months	6.88* ± 8.21	2.0 ± 6.41
Treatment	Baseline	6.85±6.81	6.76±5.76
	3 Months	1.55*±3.11	0.37**±2.15

Values are mean ±SD **p>0.001; * p>0.05.

Discussion

This study sought to investigate the effect of a novel supplementation on levels of DNA damage in HD patients. In the UK HD patients are not routinely provided with supplements, only 3.7% of HD patients in the UK receive any supplementation, unlike other countries, the Dialysis Outcomes and Practice Patterns Study [34] reported a large variation by region in the percentage of patients administered with water-soluble vitamins, ranging from 3.7% in the United Kingdom, 5.6% in Japan, 37.9% in Spain, to a high of 71.9% in the United States. Yet the use of water-soluble vitamins was associated with a substantially and significantly lower risk for mortality (RR, 0.84; $P = 0.001$) [34].

The supplement designed for this study included water-soluble vitamins, and co-factors for endogenous antioxidant enzymes (Superoxide dismutase and Glutathione peroxidase) and essential trace elements such as copper, zinc and selenium because of inadequate intake or excess removal by dialysis [34].

In the current study we observed a significant reduction in Alkaline, EndoIII, FPG and net oxidative specific FPG and EndoIII DNA damage post-intervention in the HD treatment group, while the placebo group had DNA damage levels significantly increased from baseline at 3 months, indicating the damaging high levels of oxidative stress were continuing in the untreated group.

While the alkaline comet assay can be used to determine the levels of alkaline DNA damage present within leukocytes, the modified comet assay is a much more sensitive technique which specifically measures oxidative DNA damage. This is particularly important because it is specifically oxidative DNA damage which has been strongly linked to the development of cancer [35].

The modified assay can measure oxidative DNA damage by including lesion specific enzymes such as End III and FPG since the DNA is readily digested. FPG recognises the common oxidised purine - 7, 8-dihydro-8-oxoguanine and ring opened purines; Endo III converts oxidised pyrimidines to strand breaks [35]. The results of previous investigations in this lab, showed that levels of alkaline DNA damage and oxidative-specific Endo III DNA damage were significantly increased among HD patients, compared to control participants (9). In addition, Stoyanova et al. [36] assessed a population of 253 patients with chronic kidney disease, including 77 receiving HD. HD patients had higher levels of DNA damage than those not currently on HD. The study also defined a positive correlation between DNA damage and creatinine and protein levels in plasma. Quoting reference data from Muller et al. [37] the authors concluded that their findings represented a significant increase in oxidative DNA damage. Our results are in agreement with these studies and also in accordance with a study by Stopper et al [38] who showed a significant increase of oxidative DNA damage in individuals undergoing HD treatment.

Here we show that the damaging effects of the elevated levels of oxidative stress can be ameliorated by this novel supplement. This is important given the restrictive diet HD patients need to

maintain resulting in decreased level of antioxidants are consumed (14). All these factors contribute to decreased levels of antioxidants available to repair the damage caused by increased levels of ROS.

In addition, we observed, in agreement with Stoyanova et al. [36], a positive correlation between the duration on dialysis (months) and levels of Endo III specific damage ($p=0.041$). This suggests a cumulative negative effect of increased levels of oxidative stress on Endo III DNA disruption, which may contribute to the increased cancer risk observed in this patient group. Such results reinforce the findings from this investigation and this effect is worthy of additional research as it may contribute in part, to the increased incidences of cancer and CVD within patients receiving HD treatment.

Finally, treatment with this novel supplement significantly reduced all DNA damage in the HD treatment group. This provides some evidential support for the supplementation of HD patients in the UK, in an attempt to reduce the levels of damaging oxidative stress. In addition, the placebo group continued to show a significant increase in all levels of DNA damage from baseline and compared to the treatment group.

We propose that these results provide a positive treatment regimen for HD patients. The treatment is low in cost and may protect the patients from the damaging effects of high levels of oxidative stress and the resulting DNA damage, and thus potentially reduce the mortality. However, we acknowledge this was a small group and further research is required to confirm these positive findings.

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